Total ginsenosides of Chinese ginseng induces cell cycle arrest and apoptosis in colorectal carcinoma HT-29 cells

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Abstract. Colorectal carcinoma (CRC) is the most frequent malignant disease of the gastrointestinal tract and it has a poor prognosis. The current treatment options for CRC are far from optimal; they have limited efficacy and toxic effects. Chinese ginseng (the dried root of *Panax ginseng*) is a medicinal herb, of which ginsenosides are the most effective anticancer component. The aim of the present study was to evaluate the anti-CRC effect of total ginsenosides of Chinese ginseng (TGCG), by analyzing the cellular and molecular pathways. This was done via MTT assay, morphological observation (DAPI staining), flow cytometry for cell cycle and apoptosis analyses, reverse transcription-quantitative polymerase chain reaction and western blot analysis. The results revealed that TGCG inhibited cell proliferation and induced cell cycle arrest and cell apoptosis in HT-29 cells in a dose-dependent manner. The mRNA expression of *CDK2*, *CDK4*, *CDK6*, *BAX*, *CDKN2B*, *CASP8*, *CASP3*, *TP53*, *TOP1*, *MYC*, *MDM2*, and *CCND1* and the protein expression of cyclin-dependent kinase (Cdk) 2, Cdk4, Cyclin D1, Bax, p21^{WAF1}, p27^{Kip1}, c-Myc, p15^{INK4b}, and p53 were revealed to be modulated by TGCG in HT-29 cells, and are all factors associated with DNA damage, cell proliferation, cell cycle and apoptosis. In conclusion, TGCG induced cell cycle arrest at the G_0/G_1 and G_2/M phases and induced apoptosis in HT-29 cells through the c-Myc- and p53-mediated signaling pathways, possibly in response to DNA damage. Therefore, TGCG may be regarded a promising candidate for development as an anticancer agent for the treatment of CRC.

Introduction

On account of the deteriorating living environment and unfavorable living habits, cancer becomes the leading cause of human mortality around the world (1). Among all cancers, colorectal carcinoma (CRC) is the most frequent malignant disease of the gastrointestinal tract and responsible for 600,000 deaths annually worldwide (2). It develops as the third most common cancer in men (746,000 cases, 10% of all cancers) and the second most common cancer in women (614,000 cases, 9.2% of all cancers) (2). More than 50% of all CRC cases occur in more developed regions, e.g., 345,000 new cases and 152,000 deaths were reported in the European Union (3). In some regions with previously low incidence rates, e.g., Eastern Europe and East Asia, significantly increasing numbers of CRC cases have been noted and attributed to changes in risk factors and diet towards a lifestyle common to Western countries (4). The individual risk of CRC is essentially dependent on non-modifiable dispositional factors such as age, sex, and family history as well as in principle modifiable exposure to risk factors (3). Up to one-third of the CRC risk may be attributable to hereditary factors, and another 30~50% of the CRC risk is attributable to lifestyle factors such as smoking, high consumption of red and processed meat, obesity, diabetes, and excessive consumption of alcohol (5).

Sadly enough, the prognosis of CRC patients is especially poor: Roughly two-third of CRC has been detected in an inoperable, advanced stage, when the first clinical signs occur (6). The current mainstay of treatment for CRC is surgical resection with chemotherapy and radiotherapy (7). Unfortunately, to date, the therapies are far from optimal due to their limited efficacy as well as toxic effects (8). For example, chemotherapeutic regimens are always involved in delivering the drug to both tumor and normal tissue, resulting in unexpected toxic effects such as neutropenia, anemia, hand-foot syndrome, diarrhea, gastrointestinal toxicity,

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mucositis, nausea, vomiting, fatigue, hematological disorders and liver toxicity (9). The adverse events not only worsen the patients' quality of life, but rather, cause patients to refuse further chemotherapy (10). Therefore, the need to develop more effective and safe agents for CRC treatment is urgent. In recent years, a growing interest has arisen for the therapeutical potential of natural resources to discover new anticancer agent, which is promising to provide a favorable option for CRC patients (11-14).

Traditional Chinese medicine (TCM) becomes a popular complementary and alternative medicine for cancer treatment in clinic. By using herbal medicines, TCM shows more therapeutic benefits but lesser side effect and cost than the conventional chemotherapeutics (15). As one of the most famous TCM, Chinese ginseng (the dried root of *Panax ginseng* C.A. Mey.) has been used for over 2,000 years as a medicine in China and is popularly used in more than 35 countries as food, health supplement, and natural remedy (16). It is claimed to be effective in treating cancer, including CRC (17). Many components are responsible for the anti-CRC effect of Chinese ginseng, including protopanaxadiol, Rg1, Rb1, Rg3, Rh2, and compound K. Protopanaxadiol is an active ginseng metabolite that can enhance the anticancer effect of chemotherapy on CRC (18). Ginsenoside compounds, Rg1, Rb1, Rg3, and Rh2, have been found to possess anti-CRC effect by inducing cell apoptosis and cell cycle arrest and inhibiting metastasis (19-22). Besides, ginsenoside compound K possesses not only anti-proliferative and pro-apoptotic effect but also synergistic activity with cancer cell-specific apoptosis-inducing cytokine on CRC (23). Therefore, ginsenoside compounds are a kind of ginseng component possessing anticancer effect on CRC. This study focused on total ginsenosides (combination of ginsenoside compounds) of Chinese ginseng and evaluated its effect on CRC cells from the cellular and molecular levels.

Materials and methods

Chemicals and reagents. Powders of total ginsenosides of Chinese ginseng (TGCG) (S25997; >80% of purity), ginsenoside Rb1 (Rb1) (B21050; >98% of purity), ginsenoside Re (Re; B21055; >98% of purity), ginsenoside Rd (Rd; B21054; >98% of purity), and ginsenoside Rg1 (Rg1) (B21057; >98% of purity) were obtained from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China; batch no. 2016Y08016). RPMI-1640 medium, fetal bovine serum (FBS), and 0.25% trypsin were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Annexin V:FITC apoptosis detection kit and cell cycle kit were purchased from BD Biosciences, (Franklin Lakes, NJ, USA). ProLong® Diamond Antifade Mountant with DAPI was purchased from Invitrogen; Thermo Fisher Scientific, Inc. All antibodies were purchased from Cell Signaling Technology Inc. (CST; Danvers, MA, USA). RNAiso Plus kit for real time PCR was purchased from Takara (Dalian, China).

Chemoprofile analyses. The HPLC analysis was performed on an Agilent 1260 Infinity system (Agilent Technologies, Inc., Santa Clara, CA, USA). Chromatographic separation was achieved on a Hypersil BDS-C18 column (250x4.6 mm, 5 μm; Shandon Scientific, Cheshire, UK) at temperature of 30°C. The mobile phase consisted of (A) acetonitrile and (B) 0.1% phosphoric acid solution with flow rate of 1.3 ml/min. Samples were eluted with a gradient elution system at 0~30 min (19% A), 30~35 min (19~24% A), and 35~60 min (24~60% A). The sample injection volume was 10 μl and the detection wavelength was 205 nm. The data was analyzed to determine the contents of Rb1, Re, Rd, and Rg1 in TGCG.

Cell line and culture. Human CRC cell lines (HT-29, HCT-116, and SW620) were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium containing 10% FBS at 37°C in a humidified 5% CO_2 incubator. The medium was changed daily and the cells were treated in their logarithmic growth phase.

MTT assay. MTT assay was performed to evaluate the cell viability of HT-29, HCT-116, and SW620 cells. Cells were seeded on 96-well plates with density of $5x10^3$ cells/well in 200 μl medium for 24 h and then treated with TGCG at different concentrations (0, 25, 50, 100, 200, 400 μg/ml) for 24 h (all cell lines), 48 h (HT-29), and 72 h (HT-29). Each 20 μl MTT solution (5.0 mg/ml) was added to each well and incubated at 37°C for 4 h. Then 150 μl DMSO was added in each well to dissolve the MTT formazan crystals and the optical density (OD) value was measured at 490 nm with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Inhibitory rate (%)=[1-(TB-treated OD/untreated OD)] x100%. The 50% inhibitory concentrations (IC_{50}) for 24, 48, and 72 h were calculated by regression analysis, respectively.

Cell morphology and DAPI staining. HT-29 cells were seeded on 6-well plates with density of $3x10^5$ cells/well for 24 h, followed by TGCG treatment at low, medium, and high doses for 24 h. The cells were harvested and washed with PBS thrice and then fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Then the cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min. An aliquot of the cells were mounted using ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific, Inc.) with DAPI for 3 min in dark and then washed thrice. The unstained and stained cells were observed under a fluorescence microscope (Carl Zeiss, Göttingen, Germany). Five coverslips were used as replicates of each group and the apoptotic nuclei of cells were visualized.

Flow cytometry. TGCG-induced apoptosis of HT-29 cells was determined by flow cytometry using an Annexin V/PI method, according to the manufacturer's protocol. Briefly, HT-29 cells were seeded on 6-well plates with density of $3x10^5$ cells/well for 24 h and then were treated with TGCG at low, medium, and high doses for another 24 h. Thereafter, the cells were harvested and washed twice with cold PBS, and then labeled with FITC Annexin V and PI in binding buffer. Fluorescence intensity of the cells was detected by BD FACSVerse™ flow cytometer (BD Biosciences). The apoptosis rate (%) for each treatment was calculated.

Cell cycle analysis was also performed by flow cytometry using BD cell cycle kit (BD Biosciences). HT-29 cells were

Table I. Primer sequences used for quantitative polymerase chain reaction analysis.

Gene	Forward primer	Reverse primer
β-actin	5'-CATGTACGTTGCTATCCAGGC-3'	5'-CTCCTTAATGTCACGCACGAT-3'
CASP3	5'-AGAACTGGACTGTGGCATTGAG-3'	5'-GCTTGTCGGCATACTGTTTCAG-3'
CASP8	5'-CTCCCCAAACTTGCTTTATG-3'	5'-AAGACCCCAGAGCATTGTTA-3'
CCND1	5'-CAATGACCCCGCACGATTTC-3'	5'-CATGGAGGGCGGATTGGAA-3'
CDKN2A	5'-CATGGTGCGCAGGTTCTTG-3'	5'-CGGGATGTGAACCACGAAA-3'
CDKN2B	5'-AACACAGAGAAGCGGATTTC-3'	5'-AGGTCCAGTCAAGGATTTCA-3'
CDK2	5'-GCTAGCAGACTTTGGACTAGCCAG-3'	5'-AGCTCGGTACCACAGGGTCA-3'
CDK4	5'- AAATCTTTGACCTGATTGGG-3'	5'- CCTTATGTAGATAAGAGTGCTG-3'
CDK6	5'-CTGAATGCTCTTGCTCCTTT-3'	5'-AAAGTTTTGGTGGTCCTTGA-3'
MDM2	5'-ACCTCACAGATTCCAGCTTCG-3'	5'-TTTCATAGTATAAGTGTCTTTTT-3'
MYC	5'-GCCACGTCTCCACACATCAG-3'	5'-TGGTGCATTTTCGGTTGTTG-3'
TOP1	5'-TCCGGAACCAGTATCGAGAAGA-3'	5'-CCTCCTTTTCATTGCCTGCTC-3'
TP53	5'-TCAACAAGATGTTTTGCCAACTG-3'	5'-ATGTGCTGTGACTGCTTGTAGATG-3'

seeded on 6-well plates with density of 3×10^5 cells/well for 24 h and then were treated with TGCG at low, medium, and high doses for another 24 h. The cells were harvested and washed with PBS thrice and then were stained with PI/RNase staining solution in accordance with the manufacturer's protocol of BD cell cycle kit (BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Relative mRNA expressions of targeted genes in HT-29 cells were detected by qPCR assay on an ABI QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems; Thermo Fischer Scientific, Inc.). The total RNA of the cells in each group was extracted using TRIzol reagent and synthesized to cDNA via RT. PCR reaction system had a 20.0 μl volume: 10.0 μl SYBR® Premix Ex Taq II (Tli RnaseH Plus), 0.8 μl PCR Forward Primer, 0.8 μl PCR Reverse Primer, 2.0 μl template cDNA, 0.4 μl ROX Reference Dye, and 6.0 μl ddH$_2$O. The qPCR reaction condition was set to 95˚C for 30 sec initial denaturation, 40 cycles of 95˚C for 5 sec denaturation, 60˚C for 34 sec annealing, and 72˚C for 40 sec extension. At the end of each reaction, a melting curve analysis was performed. β-actin was used as the reference gene and $2^{-\Delta\Delta CT}$ method was applied to analyze the relative mRNA expressions (Table I).

Western blot analysis. The total proteins of the TH-29 cells (1.5×10^6) were extracted using a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.1% SDS) with proteinase inhibitor cocktail (Bimake, Houston, TX, USA) for 30 min on ice. The proteins were separated by a denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 6-12%) and then transferred onto a nitrocellulose membrane (Sartorius Stedim, Goettingen, Germany). The membrane was blocked with 5% non-fat milk for 2 h, followed by overnight incubation at 4˚C with the primary antibodies [Bax, cyclin-dependent kinase (Cdk2), Cdk4, c-Myc, Cyclin D1, p15^{INK4b}, p21^{WAF1}, p27^{Kip1}, p53]. Following incubation with peroxidase-conjugated goat anti-rabbit/mouse IgG at room temperature for 2 h, proteins were visualized using

Western Lightning® Plus ECL (Perkin Elmer, Waltham, MA, USA) and detected using X-film (Kodak, Tokyo, Japan) and scanned.

Statistical analysis. Data were expressed as mean ± SD and subjected to one-way analysis of variance, followed by Fisher's least significant difference comparison. All analyses were performed using an updated version of DPS software (24). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The anti-proliferative effect of TGCG on CRC cells were determined by MTT assay. As shown in Fig. 1A, TGCG obviously inhibited the cell viability of HT-29 at a dose range of 25 to 400 μg/ml and a time range of 24 to 72 h. The inhibitory effect was increased with increasing TGCG doses at each time point and also with increasing time period at almost each dose point, indicating a time- and dose-dependent manner of TGCG. The IC$_{50}$ values were 395.79, 124.63, and 105.21 μg/ml for 24, 48 and 72 h treatment, respectively. We employed 50, 100, and 200 μg/ml as low, medium, and high doses of TGCG at 24 h for the following assays. As compared with the inhibitory effects on HCT-116 and SW620, TGCG showed a stronger effect on HT-29 (Fig. 1B).

The inhibitory effect of TGCG on TH-29 cells at morphological level was observed with DAPI staining by fluorescence microscopy. As shown in Fig. 1C, detached cells in round and shrunken shape were seen with TGCG treatment under light microscope (indicated by arrows). DAPI staining showed typical apoptotic signs on TGCG-treated cells, such as chromatin condensation, karyopyknosis and nuclear fragmentation (indicated by arrows), indicating cell apoptosis induced by TGCG.

HPLC analysis showed the chemoprofile of TGCG comprising Rb1, Rd, Re, and Rg1 (Fig. 2A). The contents of Rb1, Rd, Re, and Rg1 in TGCG are 85.23±0.65, 97.50±1.93, 153.67±1.15, and 71.27±0.50 μg/mg, respectively (Fig. 2B).
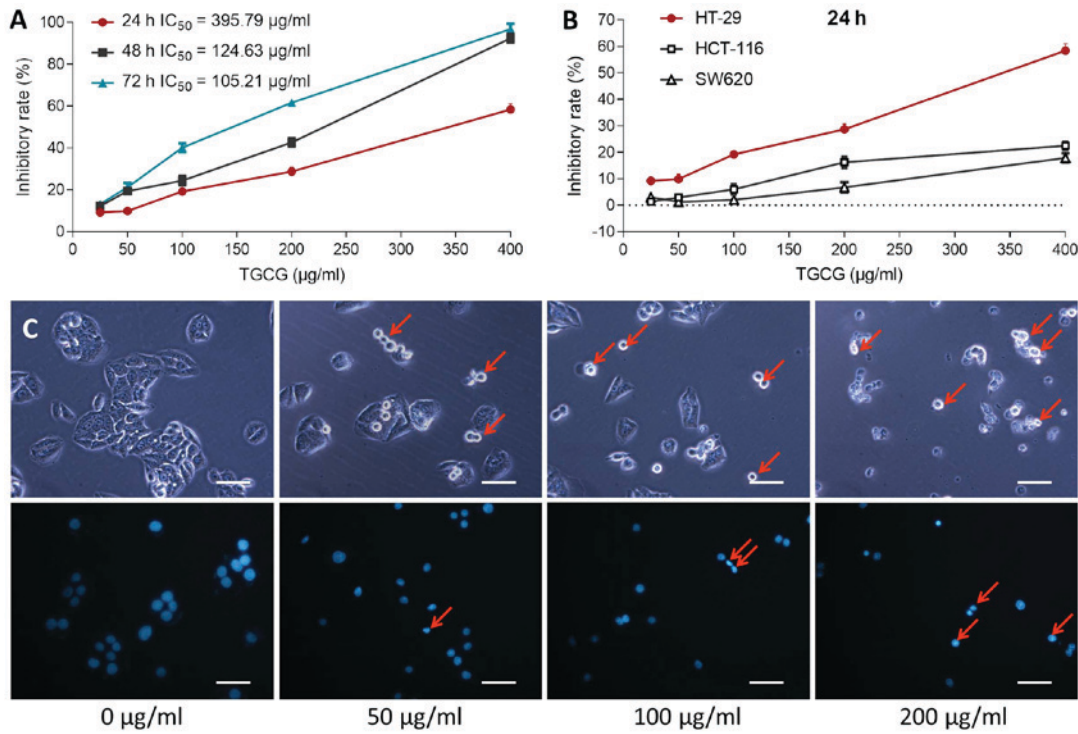
Figure 1. (A) cell viability of HT-29 cells following TGCG treatment at 24, 48 and 72 h. (B) Cell viability of HT-29, HCT-116 and SW620 cells following TGCG treatment at 24 h. (C) Morphological observation (light microscope and fluorescence microscope) of HT-29 cells following TGCG treatment. Values are presented as the mean ± standard deviation, n=5. The red arrows indicate apoptotic cells. Scale bar, 60 μm. TGCG, total ginsenosides of Chinese ginseng.
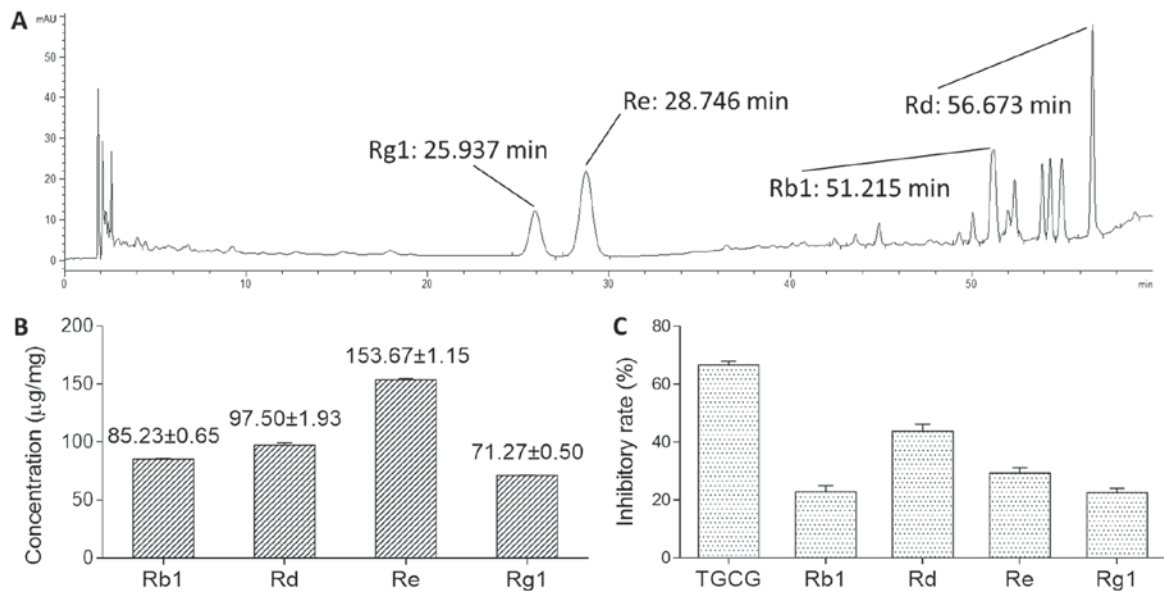

Figure 2. (A) HPLC chemoprofile and (B) concentrations of TGCG and its ginsenoside components. (C) Cell viability of HT-29 cells treated with TGCG, Rb1, Rd, Re, and Rg1 at 400 μg/ml for 24 h. TGCG, total ginsenosides of Chinese ginseng.

MTT assay indicated that TGCG at 400 μg/ml exerted stronger inhibitory effect than that of its ginsenoside component on HT-29 cells after 24 h treatment (Fig. 2C).

The apoptosis-inducing effect of TGCG on HT-29 cells was further studied by Annexin V-FITC/PI double staining assay using flow cytometry. As shown in Fig. 3, early and late apoptosis increased progressively from 1.27 to 66.45% with treatment of increasing doses of TGCG from 0 to 200 μg/ml, indicating a dose-dependent manner. Particularly, TGCG upon 100 μg/ml primarily induced early apoptosis (42.15%) in HT-29 cells.

The effect of TGCG on HT-29 cell cycle progression was performed by flow cytometry. As shown in Fig. 4, after 24 h treatment on HT-29 cells, TGCG induced obvious G_0/G_1 phase accumulation and S phase depletion in a dose-dependent manner. A visible increase of cell number in G_2/M phase was also found with TGCG treatment. These trends indicated that TGCG could induce cell cycle arrest in HT-29 cells.
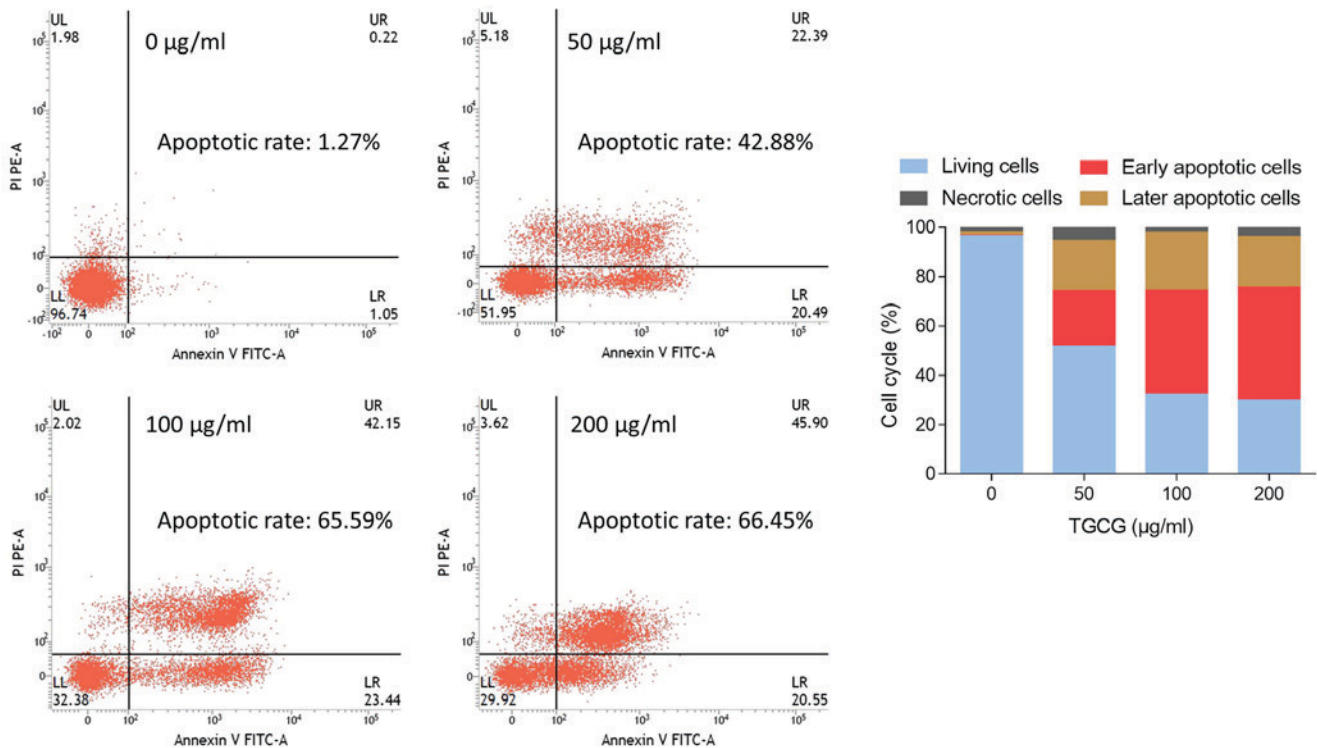
Figure 3. Flow cytometry analysis of HT-29 cell apoptosis following TGCG treatment by double staining with Annexin V-fluorescein isothiocyanate and propidium iodide. TGCG, total ginsenosides of Chinese ginseng.

The regulatory effect of TGCG on the relative expression of target genes in HT-29 cells was determined by qPCR assay. As shown in Fig. 5, with 24 h treatment, *TGCG* could significantly downregulate the expression of *CDK2*, *CDK4*, *CDK6*, *TOP1*, *MYC*, *MDM2*, and *CCND1* mRNA transcripts and upregulate the expression of *BAX*, *CDNK2B*, *CASP8*, *CASP3*, and *TP53* mRNA transcripts in HT-29 cells as compared to the untreated group. In most cases, TGCG induced a dose-dependent manner in the mRNA modulation.

The regulatory effect of TGCG on protein expression of HT-29 cells was determined by western blot assay. Proteins related to cell apoptosis and cell cycle were analyzed by different experiments under the same condition and the control (β-tubulin) was found similarly expressed in each experiment. The expression profiles of tested proteins were assembled together in Fig. 6 with one representative control. With 24 h treatment, TGCG could significantly downregulate the expression of cell cycle checkpoint proteins (Cdk2, Cdk4, and Cyclin D1) and upregulate the expression of cell apoptosis- and cell cycle related proteins (Bax, p21^{WAF1}, p27^{Kip1}, c-Myc, p15^{INK4b}, and p53) as compared to the untreated group.

Discussion

An epidemiological survey on 1,000 subjects revealed reduced risks of various cancers, including CRC, in those who received ginseng, when compared with those who did not use it (25). Many studies have been conducted on the anti-CRC effects of ginseng from its crude extracts to single components. For instance, both fermented ginseng extract and ginseng polysaccharide fraction showed anti-proliferative and anti-invasive effects on HT-29 cells (26). A steamed extract of ginseng root

induced mitochondrial damage and cell apoptosis by producing reactive oxygen species (ROS) in CRC cells (27). As a main component of ginseng, ginsenoside has been shown to exert strong anticancer activity by blocking cell cycle progression at G$_1$ phase or G$_1$/S boundary in breast and liver cancer cells through activation of p21^{WAF1}, p27^{Kip1}, and p53 (28-30). It can also induce cancer cell apoptosis by altering mitochondrial membrane integrity, releasing cytochrome *c*, and activating caspase proteases (31,32). Nevertheless, little report has determined whether ginsenoside has anti-CRC effect.

In this study, TGCG arrested cell cycle of CRC cells at G$_0$/G$_1$ and G$_2$/M phases by inhibiting cell cycle activators (c-Myc, Cdk2, Cdk4, Cdk6, and Cyclin D1) and activating cell cycle inhibitors (p15^{INK4b}, p21^{WAF1}, and p27^{Kip1}). Since Cdk2, Cdk4, Cdk6, Cyclin D1, p15^{INK4b}, p21^{WAF1}, and p27^{Kip1} could be encoded by the downstream genes of c-Myc, TGCG was concluded to induce cell cycle arrest through a c-Myc-mediated mechanism (33-35). Cyclin D1 and c-Myc are the downstream targets of Wnt/β-catenin signaling pathway, the inhibition of which causes suppression of tumor growth, epithelial mesenchymal transition (EMT), and cell motility of CRC (36). Besides, c-Myc, Cyclin D1, Cdk2, and Cdk4 are also the downstream cell cycle factors of PI3K/Akt signaling pathway in CRC, while p21 and p27 are its downstream cell cycle inhibitors (37,38). Furthermore, c-Myc and Cyclin D1 are cell proliferative effectors in the downstream of NF-κB signaling pathway in CRC (39). Therefore, Wnt/β-catenin, PI3K/Akt, and NF-κB signaling pathways might possibly participate in the action mechanism of TGCG underlying the observed effect on cell cycle and proliferation of CRC. It has been reported that ginseng extract could enhance the anti-proliferative effect of 5-fluorouracil (5-FU) on CRC cells
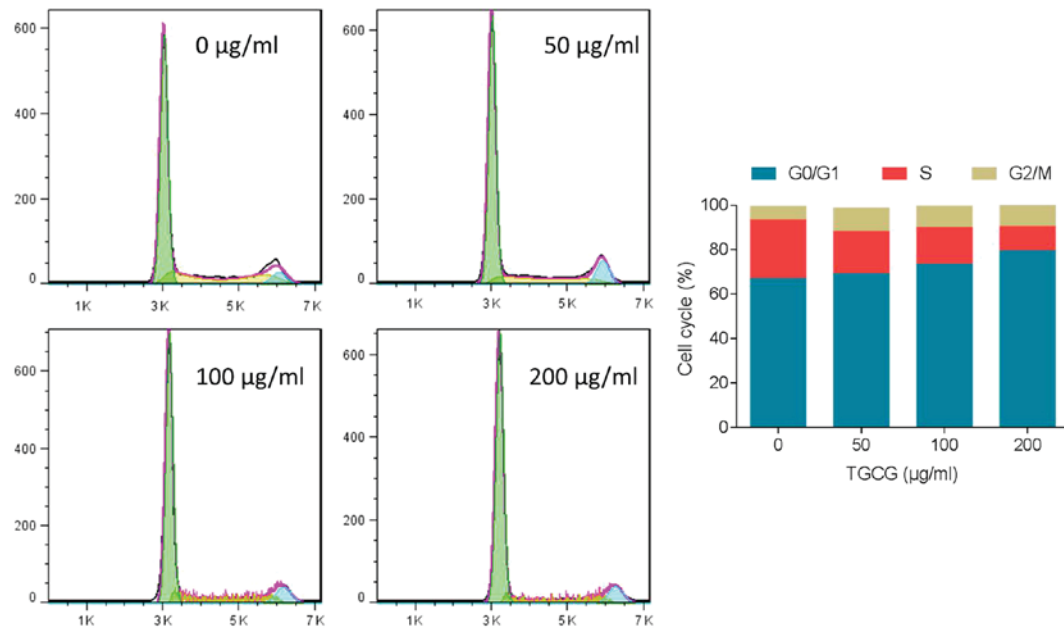
Figure 4. Flow cytometry analysis of HT-29 cell cycle progression following TGCG treatment. TGCG, total ginsenosides of Chinese ginseng.
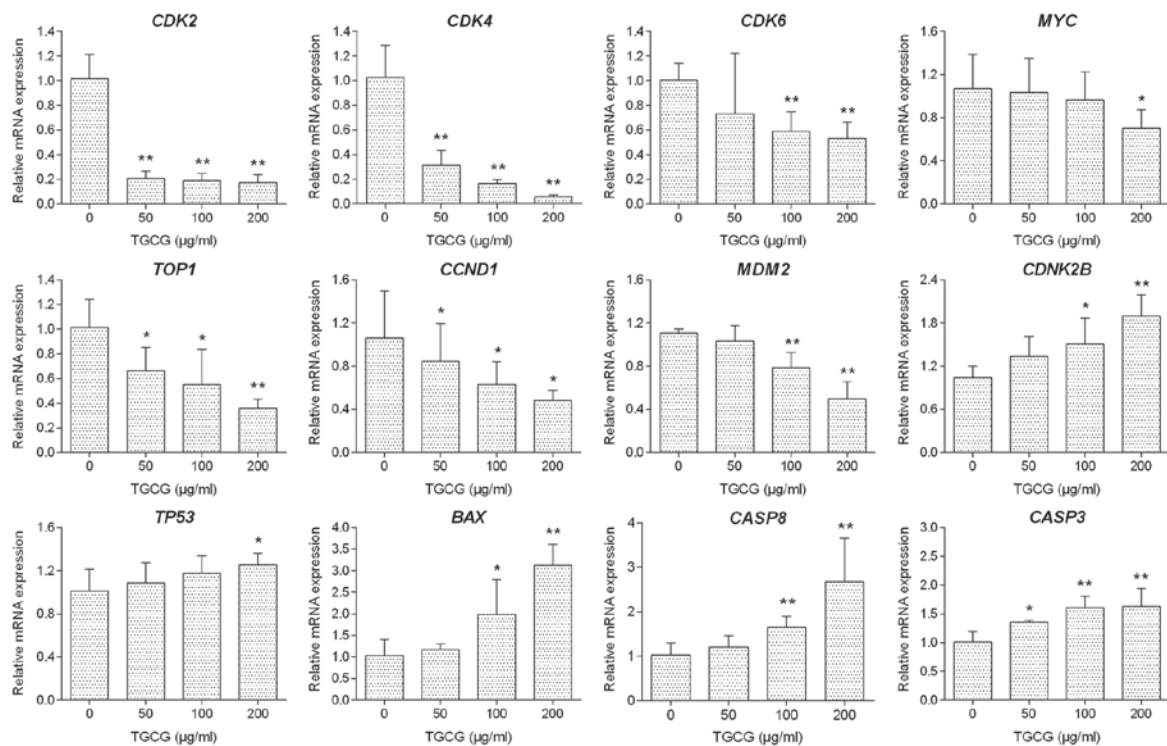

Figure 5. Relative mRNA expression of target genes in HT-29 cells following 24 h TGCG treatment. Values are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. the untreated group (0 µg/ml). TGCG, total ginsenosides of Chinese ginseng; Cdk, cyclin-dependent kinase.

and attenuate nausea and vomiting induced by chemotherapeutics, indicating an effect-enhancing and toxicity-reducing activity (40-42). 5-FU is a key drug that causes perturbation of ribosome biogenesis in CRC cells by activating ribosomal proteins and p21, leading to p21-mediated cell cycle arrest and apoptosis (43,44). It is known that c-Myc is a key regulator of ribosome biogenesis and emerging data indicate that 5-FU induces apoptosis through ribosomal protein-mediated regulation of NF-κB. In this study, TGCG has been shown

to function on c-Myc and p21, thereby indicating a possible mechanism that TGCG regulates c-Myc expression to activate p21/ribosomal protein and leads to cell cycle arrest of CRC.

It was also found that TGCG induced cell apoptosis of CRC by activating p53-mediated apoptotic pathway where *TOP1* and *MDM2* were downregulated and *TP53*, *BAX*, *CASP3*, and *CASP8* were upregulated. *TOP1* encodes DNA topoisomerase I for DNA repair during DNA synthesis and meiotic division. It is highly expressed in cancer cells making transient single-strand DNA
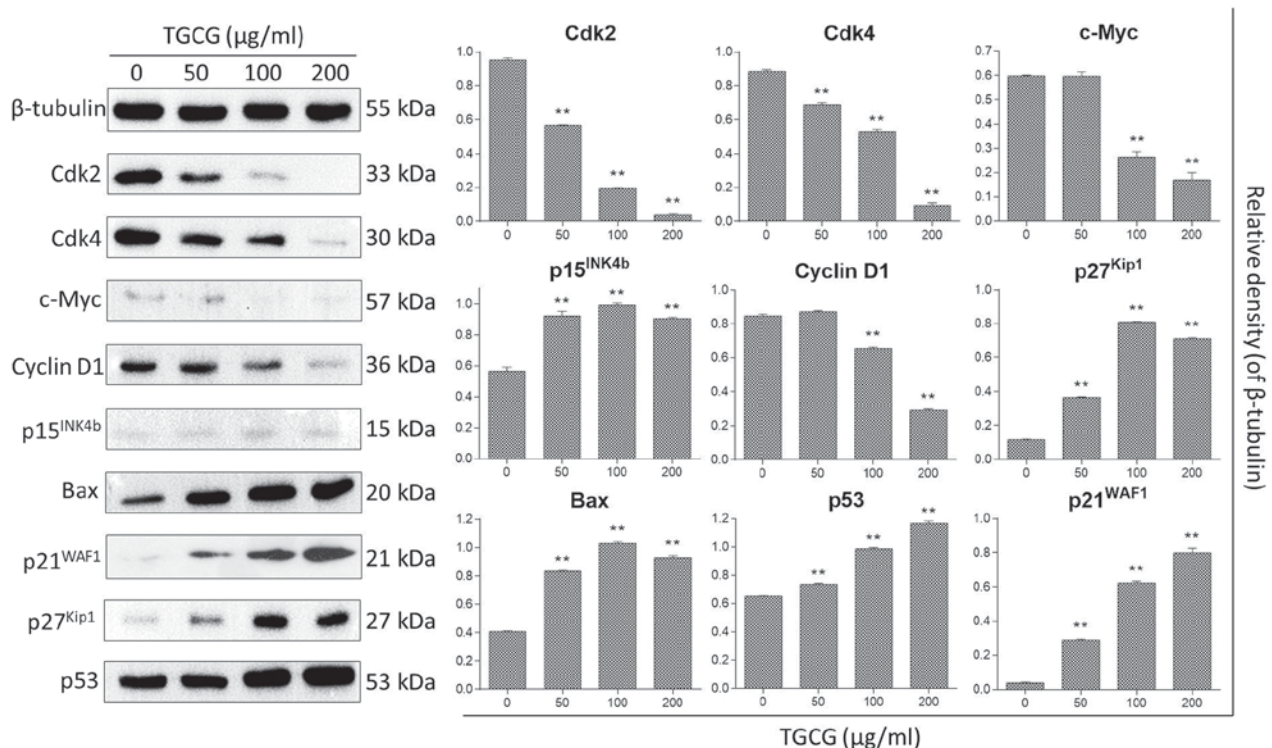
Figure 6. Western blot analysis was performed to measure protein expression in HT-29 cells following 24 h TGCG treatment. Values are presented as the mean ± standard devaiton. **P<0.01 vs. the untreated group (0 μg/ml). TGCG, total ginsenosides of Chinese ginseng; Cdk, cyclin-dependent kinase.

breaks to solve topological problems of DNA, while DNA strand breaks (DSBs) formed with inhibition of its expression (45). DSBs can trigger DNA damage with induction of apoptosis in cancer cells (46), which might be an initial event in the action of TGCG. *TP53* encodes the tumor suppressor p53 that can be activated in response to DNA damage. It is a crucial transcription factor responsible for the prevention of cancer formation due to its role in conserving stability of genome (47). When DNA suffers irreparable damage, p53 can bind DNA and initiate cell apoptosis, which in turn activates transcription of many apoptotic genes, including the members of Bcl-2 family (e.g., *BAX*) and caspase family (e.g., *CASP3* and *CASP8*) (48). However, the transcriptional activity of p53 can be inhibited by an E3 ubiquitin ligase (Mdm2) encoded by *MDM2*. Mdm2 is a negative regulator of p53 and is always overexpressed in tumor cells, which would shuttle p53 out of the nucleus and terminate p53-mediated apoptosis (49). In this study, TGCG activated p53 and inhibited *MDM2* expression to induce cell apoptosis following activation of p53-downstreamed target genes (*BAX*, *CASP3* and *CASP8*). Bax functions as an apoptosis activator tending to interact with mitochondria and release cytochrome *c* and pro-apoptotic factors to activate caspases (50). Caspase-8 encoded by *CASP8* plays an initial role in the caspase cascade which activates execution-phase of cell apoptosis. Overexpression of *CASP8* induces apoptotic cell death in tumor cells, and it most likely acts upon caspase-3. Caspase-3 encoded by *CASP3* acts as the predominant 'executioner caspase' in apoptotic cell death. It can be activated in the apoptotic cells both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways (51).

Taken together, our results indicate that TGCG induced cell cycle arrest at G_0/G_1 and G_2/M phases and apoptosis in HT-29 cells via c-Myc- and p53-associated mechanism, respectively,

possibly in response to DNA damage. Recently, increasing studies have been focused on the anti-CRC activity of the single compound of Chinese ginseng and obtained positive outcomes. We found that TGCG exerted stronger anti-CRC effect than that of each compound of Chinese ginseng (Rb1, Re, Rd, and Rg1), indicating a synergistic action of those compounds in TGCG. It makes TGCG a promising candidate for anti-CRC application and drug development due to its better effect, easier preparation procedure, and lower cost.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TL and WS were the principal scientists conducted the experiments. XD, WY and JC assisted with the experiments. LS and QY conceived, designed and revised the study and drafted the manuscript. TE made substantial contributions to the experimental design and revised the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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